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<b>(21) International Application Number:</b> PCT/EP92/00302 <b>(22) International Filing Date:</b> 11 February 1992 (11.02.92) <b>(30) Priority data:</b> P 41 04 782.6 13 February 1991 (13.02.91) DE <b>(71) Applicant (for all designated States except US):</b> INSTITUT FÜR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH [DE/DE]; Ihnestrasse 63, D-1000 Berlin 33 (DE). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> WILLMITZER, Lothar [DE/DE]; Am Kleinen Wannsee 34, D-1000 Berlin 39 (DE). SONNEWALD, Uwe [DE/DE]; Gierkezeile 34, D-1000 Berlin 10 (DE). KOSSMANN, Jens [DE/DE]; Koblenzer Strasse 1, D-1000 Berlin 31 (DE). MÜLLER-RÖBER, Bernd [DE/DE]; Lynarstrasse 5, D-1000 Berlin 65 (DE). VISSER, Richard, Gerardus, Franciscus [NL/NL]; Van Broeckhuysenstraat 9, NL-6721 TC Bennekom (NL). JACOBSEN, Evert [NL/NL]; Rizema Bosweg 77, NL-6708 DK Wageningen (NL).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), RU, SE (European patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PLASMIDS CONTAINING DNA-SEQUENCES THAT CAUSE CHANGES IN THE CARBOHYDRATE CONCENTRATION AND THE CARBOHYDRATE COMPOSITION IN PLANTS, AS WELL AS PLANT CELLS AND PLANTS CONTAINING THESE PLASMIDS  <b>(57) Abstract</b>  Plasmids are described having DNA sequences that after insertion into the genome of the plants cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants. These changes can be obtained from a sequence of a branching enzyme that is located on these plasmids. This branching enzyme alters the amylose/amylopectin ratio in starch of the plants, especially in commercially used plants.		

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Plasmids containing DNA-Sequences that cause changes in the carbohydrate concentration and the carbohydrate composition in plants, as well as plant cells and plants containing these plasmids

5

The present invention relates to plasmids containing DNA-sequences which contain information that, after insertion into a plant genome, cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants, as well as plant cells and plants containing sequences from these plasmids.

Because of the continual growth in world population, there is a continually growing demand for nutrient and raw materials. It is the task of biotechnological research to achieve a change of the content as well as yield of crops. To do this the metabolism of the plants has to be altered.

A particular interest is the possibility of using plant ingredients as renewable raw material sources e.g. for the chemical industry. This is especially of great importance for two reasons. Firstly, up to now, mineral oil and coal deposits have been the main source of raw materials for the petrochemical industry but these deposits are finite and it can be seen that alternative, renewable raw material sources must be developed.

Secondly, the present situation of agriculture in Europe and North America has lead to a surplus of crops grown for their nutritive properties. This causes obvious financial and political problems in agriculture. Alternative products for which there is a higher quantitative demand could be a solution to this problem.

Renewable raw materials can be divided into fats and oils, proteins and carbohydrates, such as mono-, di-, oligo- and

polysaccharides. The most important polysaccharides are starch and cellulose. In the EEC, the total starch production in 1987-1988 comprised maize (60%), wheat (19%) and potato (21%).

5

For an increasing use of plant starch as an industrial raw material the quality of the starch must meet the demands of the processing industry. Important considerations include the amylose to amylopectin ratio, the chain  
10 length, the branching grade of the amylopectin as well as the size of the starch granules.

The main biochemical synthetic pathways for the production of starch in higher plants are well known. Starch consists  
15 of amylose and amylopectin, in which the amylose consists of a linear  $\alpha$ -1,4-glucan and amylopectin consists of  $\alpha$ -1,4-glucans, which are connected to each other via  $\alpha$ 1,6-linkages and thus form a branched polyglucan. The so-called branching enzyme (Q-enzyme) is responsible for the  
20 introduction of the  $\alpha$ -1,6-linkage. One method for the production of starch which only has a linear  $\alpha$ -1,4-glucan structure is therefore by the inhibition of the enzymatic activity of the proteins and/or the inhibition of the biosynthesis of the branching enzyme. New biotechnology  
25 processes for the genetic alteration of dicotyledonous and monocotyledonous plants by transfer and stable installation of single isolated genes or groups of genes are known (Gasser and Fraley, Science 244, 1293-1299). The possibility of specific expression of foreign genes  
30 inserted in the plant by gene technology, primarily in potato tubers, is also known (EP 375092 and Rocha-Sosa et al., EMBO J. 8, 23-29 (1989)).

The present invention provides plasmids containing  
35 DNA-sequences which contain information that, after

insertion into a plant genome, cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants.

- 5 The invention further provides plant cells containing sequences from these plasmids which can be regenerated to whole plants, as well as plants containing sequences from these plasmids.
- 10 The term "plant" means a commercially useful plant, preferably maize, barley, wheat, rice, peas, soya beans, sugar cane, sugar beet, tomato, potato or tobacco.

- Carbohydrates which can be altered by the DNA sequences
- 15 are mono-, di-, oligo- or polysaccharides. Starch is an example of a polysaccharide which can be modified in plants and plant cells.

- With the plasmids of the invention, it is possible to
- 20 modify the amylose to amylopectin ratio of the starch in plant cells and in plants. This is possible through the presence of a branching enzyme, located on the plasmid, which has the following sequence:

	10	20	30	40	50	60
1	TCAGGAGCGGCTTTG66ATATTTCTTCCACCCAAAATCAAGAGTTAGAAAAGATGAAAG					
61	GATGAAGCACAGTTCAAGCTATTTCC6CTGTTTGGACC6ATGACAATTCGACAATGGCACC					
121	CCTAGAGGAAGATGTCAACACTGAAAATATTGGCCTCCTAAATTTGGATCCAACCTTTGGA					
181	ACCTTATCTAGATCACTTCAGACACAGAATGAAGAGATATGTGGATCAGAAAATGCTCAT					
241	TGAAAAATATGAGGGACCCCTTGAGGAATTTGCTCAAAGTTATTTAAATTTGGATTCAA					
301	CA66GAAGATG6TTG6CATAGTCTATC6TGAAATG6G6CTCCTGCTGCTCA6GAAGCAGAAGT					
361	TATTG6CGATTTCAATG6TA6GAACG6TTCTAACCACATGATGGAGAAG6ACCAAGTTGG					
421	T6TTT6GA6TATTAGAATTCCTGATGTTGACAGTAAGCCAGTCATTCCACACAACCTCCAG					
481	AGTTAAGTTTCGTTTCAAACATG6TAATG6AGTGTG66TAGATCGTATCCCTGCTTGGAT					
541	AAAGTATGCCACTG6CAGACGCCACAAAGTTG6CAGCACCATATGATGGTGTCTACTGGGA					
601	CCCACCACCTTCA6AAAAGGTACCACTTCAAATACCCTCGCCCTCCCAAACCCCGAGCCCC					
661	AC6AATCTAC6AA6CACATGTG6G6CAT6AGCA6CTCTGAGCCACGT6TAAATTCGTATCG					
721	T6AGTTT6CAGAT6AT6TTTTACCTCGGATTAAG6CAAATAACTATAATACTGTCCAGTT					

781 GATGGCCATAATGGAAACATTCTTACTATGGATCATTTGGATATCATGTTACAAACTTTTT  
841 TGCTGTGAACAATAGATATGGAAACCCGGAGGACCTAAAGTATCTGATAGATAAAACACA  
901 TAGCTTG66TTTACAG6TTCTGGTGGATGTAGTTCACAGTCATGCAAGCAATAATGTAC  
961 TGATGGCCTCAATGGCTTTGATATTGGCCAAG6TTCTCAAGAATCCTACTTTTCTGCTGG  
1021 AGAGCGAGGGTACCATAAAGTTGTGGGATAAGCAAGCTGTTCAACTATGCCAATTGGGAGGT  
1081 TCTTCGTTTCCTTCTTTCCAACCTGAGGTGGTGGCTAGAAGAGTATAACTTTGACG6ATT  
1141 TCGATTTGATGGAATAAATTCTATGCTGTATGTTTCATCATGGAATCAATATGGGATTTAC  
1201 AGGAAACTATAATGAGTATTTGAGCGAGGCTACAGATGTTGATGCTGTGGTCTATTTAAT  
1261 GTTGGCCAATAATCTGATTCACAA6ATTTTCCAGACGCAACTGTTATTGCCGAAGATGT  
1321 TTCTGGTATGCCGGGCTTAGCCGGCCTGTTTCTGAAGGGAGGAATTGGTTTTGATTACCG  
1381 CCTGGCAATGGCAATCCAGATAAGTGGATAGATTATTTAAAGAATAAGAATGATGAAGA  
1441 TTGGTCCATGAAGGAAAGTAACATCGAGTTTGACAAATAGGAGATATACAGAGAAAGTGTAT  
1501 AGCATATGCGGAGAGCCATGATCAGTCTATTGTCGGTGACAAGACCATTGCAATTTCTCCT  
1561 AATGAACAAAGAGATGTATTCTGGCATGTCTTGCTTGACAGATGCTTCTCCTGTTGTTGA  
1621 TGCAAGGAATTGCGCTTGACAAGATGATCCATTTTTTTCACAATGGCCTTGGGAGGAGAGG  
1681 GGTACCTCAATTTCTATGGGTAAACGAGTTTGCCCATCCTGAGTGGATTGACTTCCCTAGTG  
1741 AGGGCAATAATTGGA6TTATGACAAATGTAGACGCCAGTGGAACTCGCAGATAGCGAAC  
1801 ACTTGAGATACAA6TTTATGAATGCATTTGATAGAGCTATGAATTCGCTCGATGAAAAGT  
1861 TCTCATTCTCGCATCAGGAAAAACAGATAGTAAGCAGCATGGATGATGATAATAAGGTTG  
1921 TTGTGTTTGAACGTGGTGACCTGGTATTTGTATTCAACTTCCACCCAAATAACACATACG  
1981 AAGGGTATAAA6TTGGATGTGACTTGCCAGGGAAATACAGAGTTGCACTGGACAGTGATG  
2041 CTTGGGAATTTGGTGGCCATGGAAAGAGCTGGTCATGATGTTGACCATTTACATCACCAG  
2101 AAGGAATACCTGGAGTTCCAAGAAACAAATTTCAATGGTCGTCCAAATTCCTTCAAAGTGC  
2161 TGCTCTCTGCGCAACATGTGTGGCTTATTACAGAGTTGATGAACGCATGTCATAAACTG  
2221 AAGATTACCAGACAGACATTTGTAGTGAAGCTACTACCAACAGCCAATATCGAGGAAAAGTG  
2281 ACGAGAAAACCTAAAGATTGATCATCTACAAATATCAATACATCATCTACAAAAAATGCTT  
2341 ATTACAGAGTTGATGAACGCATGTCAGAAAGCTGAAGATTACCAGACAGACATTTGTAGTG  
2401 AGCTACTACTACCAACAGCCAATATCGAGGAGAGTGACGAGAACTTGATGATTGATTAT  
2461 CTACAAATATCAGTAAACATTGGTCAGACTGTTGTAGTTTCTGTTGAGGAGAGAGACAAAGG  
2521 AACTTAAAGATTACCATCTGTAAAGCATCATTA6TGATGCTGTTCCA6CTGAATGGGCTG  
2581 ATTC6GATGCAAC6TCTG66GTGAGGACTAGTCA6ATGATTGATC6ATCCTTCTACGTT  
2641 GGTGATCTCGGTCCGTGATGATGCTTCAGGGTGGTAGCATTGACTGATTGATCATAG  
2701 TTTTTTTTTTTTTTTTAAAGTATTTCTCTATGATATTATTAGCATCCAATAAATTTAC  
2761 TGGTTGTTGTACATAGAAAAAGTGATTTGATGTATGTGTTTCTCTGAAATTTTCCCA  
2821 GTTTTGGTGTCTTTGCTTTGGAGCCAA6TCTCTATATGTAATAAGAAAACCTAAGAACAA  
2881 CACATATATAAAATGTTAGTGA6ATTACCA .

The property of the branching enzyme to modify the amylose/amylopectin ratio in starch is not limited to a coding sequence exactly as it is shown here but can also be represented by slightly different nucleotid sequences.

- 5 The property of the branching enzyme is also not changed when the plasmids containing the branching enzyme, are modified in the plant cell or the plant.

10 To be active, the DNA sequence of the branching enzyme is fused to the regulatory sequences of other genes which guarantee a transcription of the DNA (coding) sequence of the branching enzyme. The DNA sequence can also be fused in an inverted direction to the regulatory sequences of other genes, whereby the 3'-end of the coding sequence is  
15 fused to the 3'-end of the promoter and the 5'-end of the coding sequence is fused to the 5'-end of the termination signal. In this way an anti-sense RNA of the branching enzyme is produced in the plant. The regulatory sequences are hereby promoters and termination signals of plant or  
20 viral genes, such as for example the promoter of the 35S RNA of the cauliflower mosaic virus or the promoter of the class I patatin-gene B 33 and the termination signal of the 3'-end of the octopine synthase gene of the T-DNA of the Ti-plasmid pTiACH5.

25 Plant cells containing sequences from these plasmids can be regenerated in known manner to complete transgenic plants. It is possible to insert simultaneously, more than one copy of these sequences into a plant cell or plant.

30

The following plasmids were deposited at the Deutsche Sammlung von Mikroorganismen (DSM) in Braunschweig, Germany on the 20th August 1990 (deposit number):

- |   |         |               |            |
|---|---------|---------------|------------|
| 5 | Plasmid | P35 S-BE      | (DSM 6143) |
|   | Plasmid | P35 S-anti-BE | (DSM 6144) |
|   | Plasmid | P33-BE        | (DSM 6145) |
|   | Plasmid | P33-anti-BE   | (DSM 6146) |

10 Description of the Figures

Figure 1 shows the restriction map of the 13.6 kb plasmid P35 S-BE. The plasmid contains the following fragments.

- |    |   |   |                                                                                                                                                                        |
|----|---|---|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 15 | A | = | Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909-7437 of the cauliflower mosaic virus. |
| 20 | B | = | Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.                                                                                   |
|    | C | = | Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the                                                                              |
| 25 |   |   | Ti-plasmid pTiACH5 from the nucleotide 11749 to 11939.                                                                                                                 |

Also shown are the cleavage sites described in Example 1.

- 30 Figure 2 shows the restriction map of the 13.6 kb plasmid P35 S-anti-BE. The plasmid contains the following fragments:

- |    |   |   |                                                                                           |
|----|---|---|-------------------------------------------------------------------------------------------|
| 35 | A | = | Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The |
|----|---|---|-------------------------------------------------------------------------------------------|



fragment contains the nucleotides 6909 to 7437 of the CaMV.

- 5        B        =        Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.
- 10       C        =        Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotides 11749-11939.

Also shown are the cleavage sites described in Example 2.

15       Figure 3 shows the restriction map of the 14.6 kb plasmid P33-BE. The plasmid contains the following fragments.

- 20       A        =        Fragment A (1526 bp) contains the DraI-DraI-fragment of the promoter region of the patatin-gene B33. The fragment contains the nucleotide positions -1512 to +14.
- B        =        Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.
- 25       C        =        Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotide positions 11749-11939.

30       Also shown are the cleavage sites described in Example 3.

Figure 4 shows the restriction map of the 14.6 plasmid P33-anti-BE. Plasmid contains the following fragments:

- 35       A        =        Fragment A (1526 bp) contains the DraI-DraI

fragment of the promoter region of the patatin gene B 33. The fragment contains the nucleotide position -1512 to +14.

- 5    B    =    Fragment B (2909 bp) contains the cDNA-fragment which codes for the branching enzyme.
- C    =    Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the
- 10       Ti-plasmid pTiACH5. The fragment contains the nucleotides 11749-11939.

Also shown are the cleavage sites described in Example 4.

- 15    In order to understand the examples forming the basis of this invention all the processes necessary for these tests and which are known per se will first of all be listed:

1.    Cloning process

- 20       The vectors pUC18/19 and pUC118, and the M13mp10 series (Yanisch-Perron et al., Gene (1985), 33, 103-119) were used for cloning.

- For plant transformation, the gene constructions were
- 25       cloned into the binary vector BIN19 (Bevan, Nucl. Acids Res. (1984), 12, 8711-8720).

2.    Bacterial strains

- The E. coli strain BMH71-18 (Messing et al., Proc. Natl. Acad. Sci. USA (1977), 24, 6342-6346) or TB1
- 30       was used for the pUC and M13 mp vectors.

- For the vector BIN19 exclusively the E. coli strain TB1 was used. TB1 is a recombinant-negative,
- 35       tetracycline-resistant derivative of strain JM101

(Yanisch-Perron et al., Gene (1985), 33, 103-119).  
The genotype of the TB1 strain is (Bart Barrel,  
personal communication): F'(traD36, proAB, lacI,  
lacZAM15),  $\Delta$ (lac, pro), SupE, thiS, recA,  
5 Sr1::Tn10(TcR).

The transformation of the plasmids into the potato  
plants was carried out by means of the Agrobacterium  
tumefaciens strain LBA4404 (Bevan, M., Nucl. Acids  
10 Res. 12, 8711-8721, (1984); BIN19 derivative).

3. Transformation of Agrobacterium tumefaciens

In the case of BIN19 derivatives, the insertion of  
the DNA into the agrobacteria was effected by direct  
15 transformation in accordance with the method  
developed by Holsters et al., (Mol. Gen. Genet.  
(1978), 163, 181-187). The plasmid DNA of transformed  
agrobacteria was isolated in accordance with the  
method developed by Birnboim and Doly (Nucl. Acids  
20 Res. (1979), 7, 1513-1523) and was separated by gel  
electrophoresis after suitable restriction cleavage.

4. Plant transformation

10 small leaves, wounded with a scalpel, of a sterile  
potato culture were placed in 10 ml of MS medium with  
25 2 % sucrose containing from 30 to 50  $\mu$ l of an  
Agrobacterium tumefaciens overnight culture grown  
under selection. After from 3 to 5 minutes gentle  
shaking, the Petri dishes were incubated in the dark  
at 25°C. After 2 days, the leaves were laid out on MS  
30 medium with 1.6 % glucose, 2 mg/l of zeatin ribose,  
0.02 mg/l of naphthylacetic acid, 0.02 mg/l of  
gibberellic acid, 500 mg/l of claforan, 50 mg/l of  
kanamycin and 0.8 % Bacto agar. After incubation for  
35 one week at 25°C and 3000 lux, the claforan

concentration in the medium was reduced by half. The regeneration and cultivation of the plants were carried out according to known processes (Rocha-Sosa et al EMBO Journal 8, 23-29 (1989)).

5

5. Analysis of genomic DNA from transgenic potato plants

The isolation of genomic plant DNA was effected in accordance with Rogers and Bendich (Plant Mol. Biol. (1985), 5, 69-76.

10

For the DNA analysis, after suitable restriction cleavage, 10 to 20 µg of DNA were analysed by means of Southern blots for the integration of the DNA sequences to be investigated.

15

6. Analysis of the total RNA from transgenic potato plants

The isolation of plant total RNA was carried out in accordance with Logemann et al. (Analytical Biochem. (1987), 163, 16-20).

20

For the analysis, 50 µg portions of total RNA were investigated by means of Northern blots for the presence of the transcripts sought.

25

7. Protein extraction

For the extraction of total protein from plant tissue, pieces of tissue were homogenised in protein extraction buffer (25 mM sodium phosphate pH 7.0, 2 mM sodium hydrogen sulphite), with the addition of 0.1 % (w/v) of insoluble polyvinylpyrrolidone (PVP).

30

After filtration through cellulose, cell detritus was centrifuged off for 20 minutes at 10,000 revolutions per minute and the protein concentration of the

35

supernatant was determined in accordance with the method developed by Bradford (Anal. Biochem. (1976)/ 72, 248-254).

5    8.    Detection of foreign proteins by means of immunological processes (Western blot)

10    The protein extracts were separated according to molecular weight by means of gel electrophoresis in SDS-PAGE (sodium dodecylsulphate polyacrylamide) gels. After SDS-PAGE the protein gels were  
15    equilibrated for from 15 to 30 minutes in transfer buffer for graphite electrodes (48 g/l of tris, 39 g/l of glycine, 0.0375 % SDS, 20 % methanol) and then transferred in a cooling chamber to a nitrocellulose filter and separated at 1.3 mA/cm<sup>2</sup> for from 1 to 2  
20    hours. The filter was saturated for 30 minutes with 3 % gelatin in TBS buffer (20 mM tris/HCl pH 7.5, 500 mM NaCl), and the filter was then incubated for 2 hours with the appropriate antiserum in a suitable  
25    dilution (1:1000 - 10000 in TBS buffer) at room temperature. The filter was then washed for 15 minutes each with TBS, TTBS (TBS buffer with 0.1% polyoxyethylene-(20)-sorbitan monolaurate) and TBS buffer. After being washed, the filter was incubated  
30    for 1 hour at room temperature with alkaline phosphatase-conjugated goat-anti-rabbit (GAR) antibodies (1:7500 in TBS). The filter was then washed as described above and equilibrated in AP buffer (100 mM tris/HCl pH 9.5, 100 mM NaCl, 5 mM  
35    MgCl<sub>2</sub>). The alkaline phosphatase reaction was started by means of the substrate addition of 70 µl of 4-nitrotetrazolium (NBT) solution (50 mg/ml of NBT in 70 % dimethyl-formamide) and 35 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml BCIP in dimethylformamide) in 50 ml of AP buffer. As a rule

the first signals were observed after 5 minutes.

9. Determination of the amylose/amylopectin ratio in starch of transgenic potato plants.

5 Leaf pieces, having a diameter of 10 mm were floated in 6% sucrose solution under continuous light for 14 hours. This light incubation induced a strong increased starch formation in the leaf pieces. After incubation, the amylose and amylopectin concentration  
10 was determined according to Hovenkamp-Hermelink et al (Potato Research 31, 241-246 (1988)).

The following examples illustrate the preparation of the plasmids according to the invention, the insertion of  
15 sequences from those plasmids into the plant cell as well regeneration of transgenic plants and the analysis of those transgenic plants.

Example 1

20 Preparation of the plasmid P35s-Be and insertion of the plasmid into the plant genome of the potato.

From a cDNA library in the expression vector pgt11, different clones were identified that cross-react with an  
25 antibody that is directed against the branching enzyme of potatoes. These clones were used to identify complete clones from a cDNA library in the HindII-position the vector pUC 19 that originate from isolated mRNA of growing potato tubers. One clone isolated in this manner had an  
30 insert size of 2909 bp of the sequence:

10 20 30 40 50 60

1 TCA6GA6C66TCTT66GATATTTCTTCCACCCCAAAATCAAGAGTTAGAAAAAGATGAAAG  
61 6ATGAA6CACAGTTCA6CTATTTCC6CTGTTTTGACC6ATGACAATTC6ACAAT66CACC  
121 CCTAGAGGAAGATGTCAACACTGAAAATATT66CCTCCTAAATTT66ATCCAACCTT66A  
181 ACCTTATCTAGATCACTTCAGACACA6AAT6AA6A6ATAT6T66ATCAGAAAATGCTCAT  
241 TGA AAAAATATGAG66ACCCCTT6A66AATTT6GCTCAA66TTATTTAAAAATTT66ATTCAA  
301 CAG6GAAGAT66TTGCATA6TCTATCGT6AAT666CTCCTGCTGCTCA6GAAGCA6AAGT  
361 TATT66C6ATTTCAAT66T6GA6AAC66TTCTAACCACAT6AT6GA6AAG6ACCA6TTT6G  
421 T6TTT66A6TATTAGAATTCCTGAT6TT6ACAGTAAGCCAGTCATTCACACAACTCCAG  
481 AGTTAA6TTTCGTTTCAAACAT66TAAT6GA6T6T666T6ATCGTATCCCTGCTT66AT  
541 AAAGTATGCCACTG6AGACGCCACAAA6TTT6CAGCACCATATGAT6GT6TCTACT66GA  
601 CCCACCACCTTCAGAAA6GTACCACTTCAAATACCCTCGCCCTCCCAAACCCGAGCCCC  
661 ACGAATCTAC6AA6CACAT6T66CAT6AGCAGCTCT6AGCCACGT6TAAATTCGTATCG  
721 T6A6TTT6CAGAT6AT6TTTTACCTCG6ATTAAGGCAAATAACTATAATACT6TCCA6TT  
781 6AT66CCATAAT6GAACATTCTTACTAT66ATCATTT66ATATCAT6TTACAAACTTTTT  
841 T6CTGT6AGCAATAGATAT6GA AACCC6GA6GACCTAAAGTATCTGATAGATAAAGCACA  
901 TAGCTT666TTTACAG6TTCT66T66AT6TAGTTCACA6TCAT6CAAGCAATAATGTCAC  
961 T6AT66CCTCAAT66CTTTGATATT66CCAAG6TTCTCAAGAATCCTACTTTTAT6CT6G  
1021 AGAGCGAG66TACCATAA6TT6T66GATAGCA66CT6TTCAACTAT6CCAATT66GA6GT  
1081 TCTTCGTTTCCTTCTTTCCAACCTGAG6T66T66CTAGAAAGATATAACTTTGACG6ATT  
1141 TCGATTT6AT6GAATAACTTCTAT6CTGTAT6TTTCATCAT6GAATCAATAT66GATTTAC  
1201 AGGAAACTATAAT6AGTATTTAGCGAGGCTACAGAT6TTGAT6CTGT66TCTATTTAAT  
1261 GTT66CCAATAATCT6ATTCACAA6ATTTTCCAGACGCAACTGTTATT66CCAAGAT6T  
1321 TTCT66TAT6CC666CCTTAGCC66CCT6TTTCTGAG6GAG6AATT6GTTTT6ATTACC6  
1381 CCT66CAAT66CAATCCAGATAAGT66ATAGATTATTTAAAGAATAAGAAT6AT6AAGA  
1441 TT6GTCCAT6AA6GA6TAACATCGAGTTT6ACAAATAGGAGATATACAGAGAAGTGTAT  
1501 AGCATAT6C66AGAGCCAT6ATCA6TCTATT6TC66T6ACAAGACCATT6CATTTCTCCT  
1561 AATGAACAAAAGAGAT6TATTCTGGCAT6TCTTGCTT6ACA6ATGCTTCTCCTGTT6TTGA  
1621 T6CA6GAATT6C6CTT6ACAA6AT6ATCCATTTTTTTTCACAATGGCCTT66GA6GA6G6  
1681 66TACCTCAATTTTAT66GTAAAC6AGTTTGGCCATCCTGAGTGGATT6ACTTCCCTAGTG  
1741 AG66CAATAATT6GA6TTATGACAAAT6T6ACGCCAGT6GAACCTCGCAGATAGC6AAC  
1801 ACTT6AGATACAA6TTTATGAATGCATTT6ATAGAGCTATGAATTCGCTCGATGAAAA6T  
1861 TCTCATTCCTG6CATCA6GAAAA6A6ATA6TAAGCA6CAT66ATGATGATAATAAG6TTG  
1921 TT6T6TTT6AAC6T66T6ACCT66TATTTGTATTCAACTTCCACCCAAATAACACATACG

10 20 30 40 50 60  
1981 AA6GGTATAAA6TT6GATGTGACTTGCCAGG6AA6TACAGAGTTGCACTG6ACAGTGATG  
2041 CTT6G6AATTT6GT6GCCATG6AAGAGCT6GTCATGATGTT6ACCATTTACATCACCAG  
2101 AA6GAATACCT6GA6TTCCAGAAA6AAATTTCAAT6GTGTC6AAATTCCTTCAAA6TG  
2161 TGTCTCCTGCG6AACATGTGT6GCTTATTACAGAGTTGATGAACG6CATGTCATAAACTG  
2221 AAGATTACCA6ACAGACATTTGTAGTGAGCTACTACCAACAGCCAATATCGAGGAAA6TG  
2281 ACGAGAAA6CTTAAAGATTCATCATCTACAAATATCAGTACATCATCTACAAAAAATGCTT  
2341 ATTACAGAGTTGATGAACG6CATGTCAGAA6CTGAA6ATTACCA6ACAGACATTTGTAGTG  
2401 AGCTACTACTACCAACAGCCAATATCGAGGAGA6TGACGAGAAA6CTTGATGATTCATTAT  
2461 CTACAAATATCAGTAACATTG6TCAGACTGTTGTAGTTTCTGTTGAGGAGAGAGACAA6G  
2521 AACTTAAAGATTACCATCTGTAA6CATCATTAGTGATGCTGTTCCAGCTGAATG6GCTG  
2581 ATTC6GATGCAAACGTCTG6G6GTGAGGACTAGTCA6ATGATTGATCGATCCTTCTACGTT  
2641 GGTGATCTCGGTCCGTG6CATGATGTCTTCAGG6TGGTAGCATTGACTGATTG6CATCATAG  
2701 TTTTTTTTTTTTTTTTAAAGTATTTCTCTATG6CATATTATTAGCATCCAATAAATTTAC  
2761 TG6TTGTTGTACATAGAAAAA6TGCAATTTG6CATGTATGTGTTTCTGAAATTTTCCCCA  
2821 GTTTT6GTGCTTTG6CCTTTGAGG6CAAGTCTCTATATGTAATAAGAAA6CTAA6AACAAT  
2881 CACATATATAAAATGTTAGTA6ATTACCA .



The 2909 bp long c-DNA contained in this clone was used for the next examples and is called cBE.

5 For the preparation of a plasmid p35s-BE, this cDNA was provided with the promoter of the 35s-RNA of the cauliflower mosaic virus as well as the polyadenylation signal of the octopine synthase gene of the Ti-plasmid pTiACH5. For this the orientation of the C-DNA coding for  
10 the branching enzyme was chosen in such a way that the coding strain will be readable (sense-orientation). The plasmid p35s-BE has a size of 13.6 kb and comprises the three fragments A, B and C which were cloned into the cleavage sites of the polylinker of BIN19.

15

Fragment A (529 bp) contains the 35s promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909 to 7437 of the CaMV (Franck et al., Cell  
21, 285-294). It was isolated as EcoRI-KpnI-fragment from  
20 the plasmid pDH51 (Pietrzak et al, Nucleic Acids Research 14, 5857-5868) and was cloned between the EcoRI-KpnI-cleavage position of the polylinker of the plasmid BIN 19.

Fragment B contains a 2909 bp cDNA fragment cBe which  
25 codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment of the vector pUC 19 and was cloned into the SmaI-position of the polylinker of BIN 19 after filling-in of the Hind-III-position with DNA polymerase. For this the orientation of the cDNA was chosen in such a way that the  
30 coding strand is readable and a sense-RNA is formed. The cleavage sites BamHI/XbaI and PstI/SphI originate from the polylinker of pUC 19. The cleavage sites BamHI/XbaI/SalI/PstI originate from the polylinker of BIN 19. The two  
EcoRI cleavage sites located on the fragment B are  
35 internal cleavage sites of the fragment.

- Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J. 3, 835,846), nucleotides 11749-11939, which are isolated as PvuII-HindIII fragment from the plasmid
- 5 PAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209-213) and were then cloned onto the PvuII cleavage site between the SphI-Hind-III cleavage site of the polylinker of BIN 19, after addition of SphI linkers (see Fig 1).
- 10 The plasmid p35s-BE was transferred into potatoes with the help of the agrobacterial system. After this whole plants were regenerated. Protein extracts isolated from tubers of these plants were tested for the existence of the
- 15 tubers of these plants were tested for the content of amylose and amylopectin.

#### Example 2

- Preparation of the plasmid p35s-anti-BE and introduction
- 20 of the plasmid the plant genome of potato.

- In a similar manner to that described in Example 1, the plasmid p35s-anti-BE was prepared, but the orientation of the designated cDNA of the branching enzyme was inverted
- 25 relative to the 35 S promotor. The plasmid p35s-anti-BE has a size of 13.6 kb and comprises the three fragments A, B and C which were cloned in the cleavage sites of the polylinker of BIN19.

- 30 Fragment A (529 bp) contains the 35s promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909 to 7437 of the CaMV (Franck et al. Cell 21, 285-294), and was isolated as EcoRI-KpnI-fragment from the plasmid pDH51 (Pietrzak et al Nucleic Acids Research
- 35 14, 5857-5868) and cloned between the EcoRI-KpnI-cleavage

site of the polylinker of the plasmid BIN 19.

Fragment B contains the 2909 bp cDNA fragment cBE which codes for the branching enzyme. It was cut from the  
5 HindIII-SmaI-fragment of the vector pUC 19 and cloned in the SmaI-position of the polylinker BIN 19 after filling in of the HindIII-position with DNA polymerase. The orientation was chosen in such a way that the non-coding strand is readable and an anti-sense-RNA is formed. The  
10 cleavage sites SphI, PstI and XbaI, BamHI, SmaI originate from the polylinker pUC 19. The cutting positions BamHI/XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI cleavage sites contained on the fragment B are internal cleavage sites of this fragment.

15 Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the TI-plasmid pTiACH5 (Gielen et al EMBO J 3, 835-846), nucleotides 11749-11939, which were isolated as PvuII-HindIII-fragment from the plasmid pAGV  
20 40 (Herrera-Estrella et al (1983), and which were cloned between the SphI-HindIII-cleavage position of the polylinker of BIN 19 after addition of Sph-I-linkers to the Pvu-II-cleavage position (see Fig 2).

25 The plasmid p35s-anti-BE was transferred into potatoes using the agrobacterial system. After this whole plants were regenerated.

30 Protein extracts, which had been isolated from tubers of these plants, were tested for the existence of the branching enzyme using the western blot analysis. Tubers of these plants were also tested for the content of amylose and amylopectin.

Example 3Preparation of the plasmid p33-BE and introduction of the plasmid into the plant genome of the potato.

5 In a similar manner to that described in Example 1, the plasmid p33-BE was prepared, but replacing the 35s promoter with the promoter of the class I patatin-gene B33 (Rocha-Sosa et al EMBO J 8 23-29). The plasmid p33-Be has a size of 14.6 kb and consists of the three fragments A, B  
10 and C that were cloned into the cleavage position of the polylinker of BIN 19.

Fragment A contains the DraI-DraI-fragment (position -1512 to position +14) of the promoter region of the patatin-  
15 gene B33 (Rocha-Sosa et al EMBO J 8. 23-29), which was first of all cloned into the SacI-position of the polylinker of pUC 18. For this the overhanging 3'- end of the Sac-I-cleavage site had been rendered blunt by T4-DNA polymerase. After this the EcoRI-BamHI-fragment was  
20 inserted between the EcoRI-BamHI-position of the polylinker of BIN 19.

Fragment B contains the 2909 bp cDNA fragment cBE which codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment from the vector pUC 19 and was cloned into  
25 the SmaI-position of the polylinker of BIN 19 after the HindIII-position was filled in with DNA polymerase. For this the orientation of the cDNA was chosen in such a way that the coding strand was readable and a sense-RNA was  
30 formed. The cleavage sites BamHI/XbaI and PstI/SphI originate from the polylinker of pUC 19. The cutting positions BamHI/XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI-cleavage sites contained on the fragment B are internal cleavage sites of  
35 this fragment.

Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid PtiACH5 (Gielen et al EMBO J 3, 835-846, Nucleotide 11749-11939), which was isolated as Pvu-II-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209-213) and which was cloned between the sphI-HindIII-cleavage site of the polylinker of BIN 19 after addition of SphI-linkers to the PvuII-cleavage site.

10 The plasmid p33-BE was transferred into Agrobacterium tumefaciens and used for the transformation of potato plants.

#### Example 4

15 Preparation of the plasmid p33-anti-BE and introduction of plasmid into the plant genome of potato.

In a similar manner to that described in Example 2, plasmid p33-anti-BE was prepared but replacing the 20 35S-promoter with the promoter of the class I patatin-gene B33 (Rocha-Sosa et al EMBO J 8, 23-29). The plasmid p33-anti-Be has a size of 14.6 kb and consists of three fragments A, B and C which were cloned into the cleavage sites of the polylinker of BIN 19.

25 Fragment A contains the DraI-DraI-fragment (position -1512 to position +14) of the promoter region of the patatin-gene B33 (Rocha-Sosa et al EMBO J 8, 23-29) which was firstly cloned into the SacI-position of the polylinker of 30 pUC 18. The overhanging 3'-ends of the SacI-cleavage site were rendered blunt by T4-DNA polymerase. After this the fragment was inserted as EcoRI-BamHI-fragment between the EcoRI-BamHI-position of the polylinker of BIN 19.

35 Fragment B contains the 2909 bp cDNA fragment cBE which

codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment from the vector pUC 18 and after filling in the HindIII-position with the DNA polymerase, it was cloned into the SmaI-position of the polylinker of BIN 19. For this the orientation of the cDNA was chosen in such a manner that the non-coding strand was readable and anti-sense-RNA could be formed. The cutting positions SphI, PstI and XbaI, BamHI, SmaI originate from the polylinker of pUC 19. The cutting positions BamHI/ XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI cleavage sites which are located on the fragment B are internal cleavage sites of the fragment.

Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J 3, 835-846), Nucleotides 11749-11939), which had been isolated as PvuII-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983), Nature 303, 209-213) and which was cloned between the SphI-HindIII-cleavage site of the polylinker of BIN 19 after addition of SphI-linkers to the PvuII cleavage sites.

The plasmid p33-anti-BE was introduced in Agrobacterium tumefaciens and was used for the transformation of potato plants.

#### Example 5

The nucleotides 166-2909 of the 2909 bp cDNA sequence described in Example 1, that codes for the branching enzyme in the HindII-cleavage site of the cloning vector pUC 19 were inserted into the corresponding cleavage sites of the polylinker of the cloning vector pUC 18. This makes possible a fusion of the N-end of the  $\alpha$ -peptide of the  $\beta$ -galactosidase located on the vector with a part of the branching enzyme. The functionality of the resulting

- fusion protein was tested in a mutant of Escherichia coli (KV 832) which is deficient in the branching enzyme (Kiel et al Gene 78, 9 - 17). Cells transformed with this construction were plated out on YT-agar plates containing
- 5 0.5% glucose. The resulting colonies were stained with Lugolscher solution. The transformed plant cells showed a yellow-red colour in contrast to the blue coloured un-transformed plant cells which indicates the branching activity of the fusion protein (Kiel et al Gene 78, 9-17).
- 10 An over-production of this protein in Escherichia coli enables the use as technical enzyme.

Claims

1. A plasmid that contains a DNA sequence that contains information that causes changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants, after insertion into the plant genome.
2. A plasmid according to Claim 1 characterised in that the DNA sequence is the coding sequence of a branching enzyme.
3. A plasmid according to Claim 2 characterised in that the branching enzyme is an enzyme having the following sequence:

10                      20                      30                      40                      50                      60

1    TCAGGAGCGGCTTTGGGATATTTCTTCCACCCCAAAATCAAGAGTTAGAAAAGATGAAAG  
61    GATGAAGCACAGTTCAAGCTATTTCCGCTGTTTTGACCGATGACAATTCGACAATGGCACC  
121   CCTAGAGGAAGATGTCAACACTGAAAAATATTGGCCTCCTAAATTTGGATCCAACCTTTGGA  
181   ACCTTATCTAGATCACTTCAGACACAGAATGAAGAGATATGTGGATCAGAAAATGCTCAT  
241   TGA AAAAATATGAAGGACCCCTTGAAGGAATTTGCTCAAGGTTATTTAAATTTGGATTCAA  
301   CAGGGAAGATGGTTGCATAGTCTATCGTGAATGGGCTCCTGCTGCTCAGGAAGCAGAAAT  
361   TATTGGCGATTTCAATGGTAGGAACGGTTCTAACCACATGATGGAGAAAGGACCAAGTTTG  
541   AAAGTATGCCACTGCAGACGCCACAAAGTTTGAAGCACCATATGATGGTGTCTACTGGGA  
601   CCCACCACCTTCAGAAAAGTACCCTTCAAATACCCTCGCCCTCCCAACCCGAGCCCC  
661   ACGAATCTACGAAGCACATGTGCGCATGAGCAAGCTCTGAGCCACGTGTAAATTCGTATCG  
721   TGAAGTTGAGATGATGTTTTACCTCGGATTAAAGGCAATAACTATAATACTGTCCAGTT  
781   GATGGCCATAATGGAACATTCTTACTATGGATCATTTGGATATCATGTTACAAACTTTTT  
841   TGCTGTGAGCAATAGATATGGAACCCGGAAGGACCTAAAGTATCTGATAGATAAAGCACA  
901   TAGCTTGGGTTTACAGGTTCTGGTGGATGTAGTTCACAGTCATGCAAGCAATAATGTAC  
961   TGATGGCTCAATGGCTTTGATATTGGCCAAAGTTCTCAAGAATCCTACTTTTCATGCTGG  
1021   AGAGCGAGGATACCATAAGTTGTGGGATAGCAAGGCTGTTCAACTATGCCAATTGGGAGGT



1081 TCTTC6TTTCCTTCTTTCCAACCTTGAGGTGGTGGCTAGAAGAGTATAACTTTGACGGATT  
1141 TC6ATTTGATGGAATAACTTCTATGCTGTATGTTTCATCATGGAATCAATATGGGATTTAC  
1201 A6GAAACTATAATGAGTATTTCA6C6A66CTACAGATGTTGATGCTGTGGTCTATTTAAT  
1261 GTTGGCCAATAATCTGATTCACAA6ATTTTCCAGACGCAACTGTTATTGCCGAA6ATGT  
1321 TTCTGGTATGCCGGGCTTA6CCGGCTGTTTCTGA6GGAGGAATTGGTTTTGATTACCG  
1381 CCTGGCAATG6CAATCCCA6ATAAGTGGATAGATTATTTAAAGAATAA6AATGATGAAGA  
1441 TTGGTCCATGAA6GAAGTAACATCGAGTTTGACAAATAGGAGATATACAGAGAA6GTAT  
1501 AGCATATGCGGAGAGCCATGATCAGTCTATTGTCGGTGACAAGACCATTGCATTTCTCCT  
1561 AATGAACAAA6A6ATGATTCTGGCATGTCTTGGTGGACAGATGCTTCTCCTGTTGTTGA  
1621 TGACGGAATTGCGCTTGACAA6ATGATCCATTTTTTTCACAAATGGCCTTGGGGA6GA6G  
1681 GGTACCTCAATTTTCATGGGTAA6GAGTTTGGCCATCCTGA6TGGATTGACTTCCCTA6TG  
1741 AGGGCAATAATTGGA6TTATGACAAATGTA6ACGCCAGTGGAACTTGGCAGATAGCGAAC  
1801 ACTTGAGATACAA6TTTATGAATGCATTTGATAGAGCTATGAATTCGCTCGATGAAAAGT  
1861 TCTCATTCCTGCGATCAGGAAAACAGATAGTAAGCAGCATGGATGATGATAATAA6GTTG  
1921 TTGTGTTTGAACGTGGTGACCTGGTATTTGTATTCAACTTCCACCCAAATAACACATAGC  
1981 AAGGGTATAAA6TTGGATGTGACTTGGCAGGGAA6GTACA6AGTTGCACTGGACAGTGTG  
2041 CTTGGGAATTTGGTGGCCATGGA6AGCTGGTCATGATGTTGACCATTTCACATCACCAG  
2101 AAGGAATACCTGGAGTTCCAGAAAACAAATTTCAATGGTGGTCCAAATTCCTTCAAAGTGC  
2161 TGTCTCCTGCGCGAACATGTGTGGCTTATTACAGAGTTGATGAACGCATGTCATAAACTG  
2221 AAGATTACCAGACAGACATTTGTAGTGA6CTACTACCAACAGCCAATATCGAGGAAAAGT  
2281 ACGAGAAACTTAAAGATTCATCATCTACAAATATCAGTACATCATCTACAAAAAATGCTT  
2341 ATTACAGAGTTGATGAACGCATGTGAGAA6CTGAA6ATTACCAGACAGACATTTGTAGTG  
2401 AGCTACTACTACCAACAGCCAATATCGAGGAGAGTGACGAGAAACTTGATGATTCAATTAT  
2461 CTACAAATATCAGTAACATTGGTCAGACTGTTGTAGTTTCTGTTGAGGAGAGAGACAA6G  
2521 AACTTAAAGATTACCATCTGTAA6CATCATTAGTGTGCTGTTCCAGCTGAATGGGCTG  
2581 ATTCGGATGCAAA6CTCTGGGGTGAGGACTAGTCAGATGATTGATCGATCCTTCTACGTT  
2641 GGATGATCTCGGTCCTGTCATGATGTCTTCAGGGTGGTAGCATTGACTGATTGCATCATAG  
2701 TTTTTTTTTTTTTTTTAA6TATTTCTCTATGTCATATTATTA6CATCCAATAAATTTAC  
2761 TGGTTGTTGTACATAGAAAAA6TGCAATTTGCATGTATGTGTTTCTGAAATTTTCCCA  
2821 GTTTTGGTGGCTTTGGAGCCAA6TCTCTATATGTAATAAGAAA6CTAAGAA6CAAT  
2881 CACATATATAAAATGTTAGTAGATTACCA .

4. A plasmid according to any one of the preceding claims, characterised in that the carbohydrates are mono-, di-, oligo- or polysaccharides.
- 5 5. A plasmid according to Claim 4 characterised in that the polysaccharide is starch.
6. A plasmid according to Claim 3 characterised in that the branching enzyme alters the amylose/amylopectin ratio of the starch in plant cells and in plants.  
10
7. A plasmid according to Claims 2 or 3 characterised in that the DNA sequence of the branching enzyme is fused to the regulatory sequences of other genes that ensures a transcription of the branching enzyme coding DNA sequence.  
15
8. A plasmid according to Claim 7 characterised in that the DNA sequence of the branching enzyme is fused in inverted direction to the regulatory sequence of other genes thereby the 3'-end of the coding sequence is fused to the 3'-end of the promoter and the 5'-end of the coding sequence is fused to the 5'-end of the termination signal that gives an anti-sense RNA in the plant produced by the branching enzyme.  
20  
25
9. A plasmid according to Claims 7 or 8 characterised in that the regulatory sequences are promoters and termination signals of plant or viral genes.  
30
10. A plasmid according to Claim 9 characterised in that the promoter is a promoter of the 35s RNA of the cauliflower mosaic virus and the termination signal is the 3'-end of the octopine-synthase-gene of the T-DNA of the Ti-plasmid pTiACH5.  
35

11. A plasmid according to Claim 9 characterised in that the promoter is a promoter of the class I patatin-gene B33.
- 5 12. Plasmid P35 S-BE (DMS 6143)
13. Plasmid P35 S-anti-BE (DSM 6144)
14. Plasmid P33-Be (DSM 6145)
- 10 15. Plasmid P33-anti-Be (DSM 6146)
16. A plant that contains a sequence of at least one plasmid according to any one of Claims 1 to 15.
- 15 17. A plant according to Claim 16 characterised in that the plants are commercially used plants such as maize, barley, wheat, rice, pea, soya bean, sugar cane, sugar beet, tomato, potato or tobacco.
- 20 18. Use of the plasmids claimed in any one of claims 12 to 15, for the production of transgenic plants in which the amylose/amylopectin ratio of the starch is modified.
- 25 19. Use of the plasmids according to Claim 18 characterised in that the plants are commercially used plants.
- 30 20. Use of the plasmids according to Claim 19 characterised in that the plants are maize, barley, wheat, rice, pea, soya bean, sugar cane, sugar beet, tomato, potato and tobacco.

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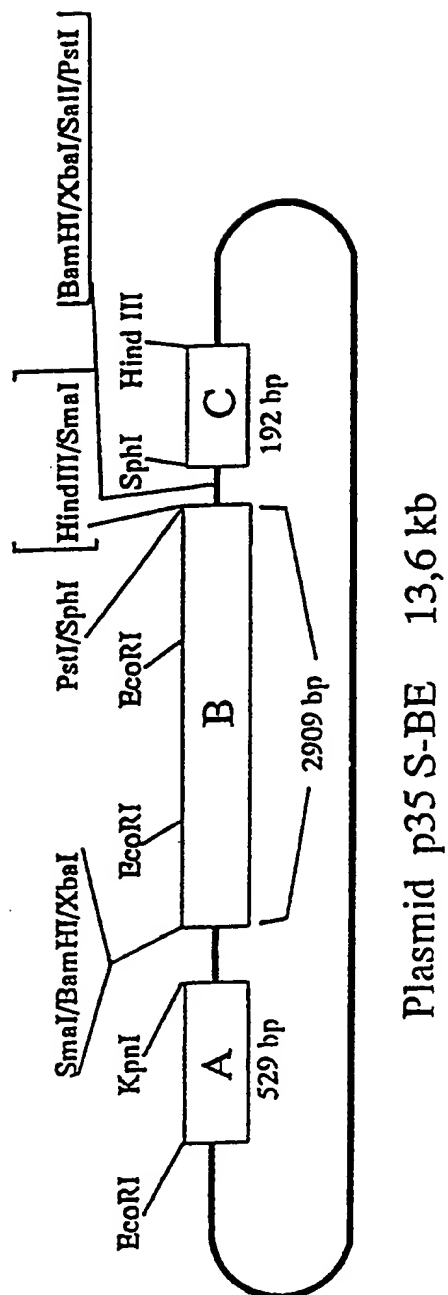
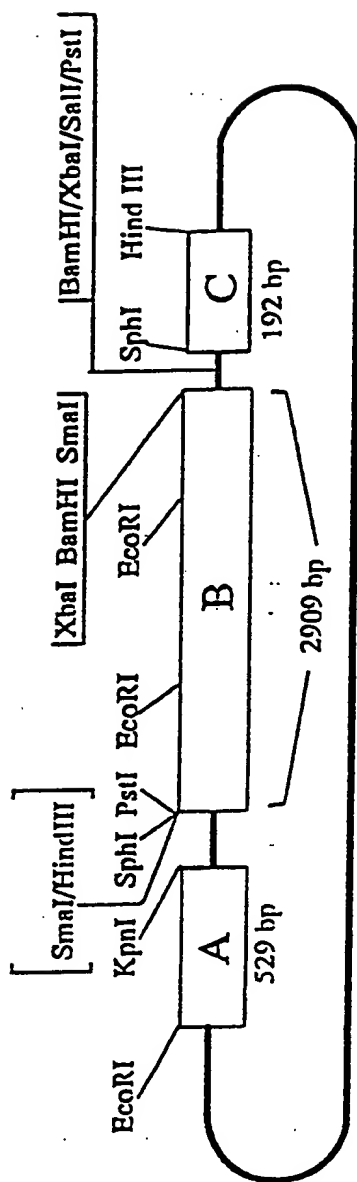


Fig. 1

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Plasmid p35 S-anti-BE 13,6 kb

Fig. 2

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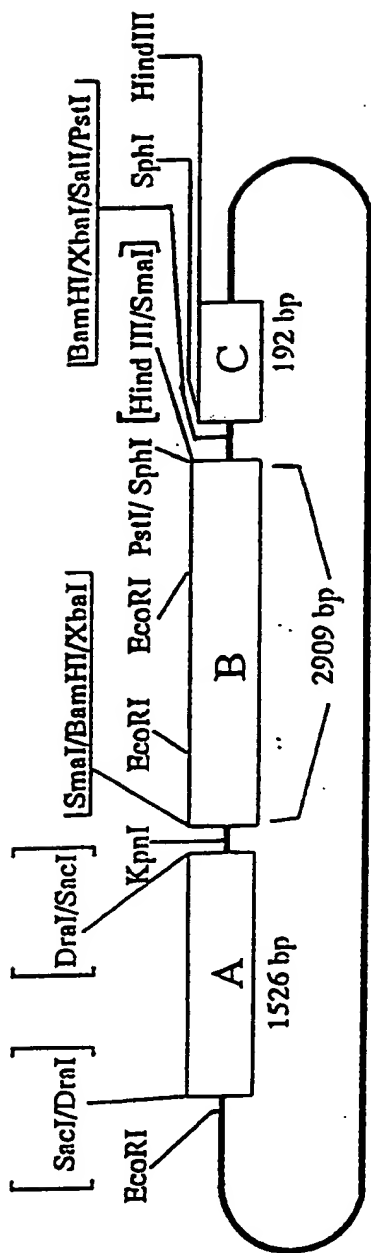
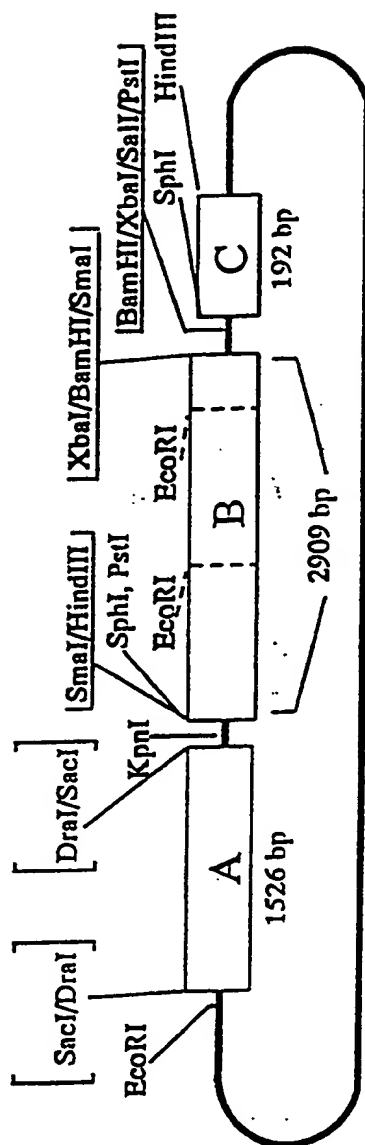


Fig. 3

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Plasmid p33 - anti-BE 14,6 kb

Fig. 4

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/82;	C12N15/54;	C12N9/10; A01H5/00
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; A01H	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	CELL. vol. 60, 12 January 1990, CAMBRIDGE, MA US pages 115 - 122; BHATTACHARYYA, M. K., ET AL.: 'The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme'	1,2,4-6
Y	see the whole document	3,7,9, 16,17
Y	WO,A,9 012 084 (DNA PLANT TECHNOLOGY) 18 October 1990 see page 9, line 17	9,16,17
<p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
15 MAY 1992	27. 05. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	MADDOX A. D.	



III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
Y	<p>PLANT PHYSIOLOGY. vol. 90, 1989, ROCKVILLE, MD, USA. pages 75 - 84; VOS-SCHERPERKEUTER, G. H., ET AL.: 'Immunological comparisin of the starch branching enzymes from potato tubers and maize kernels' see the whole document</p> <p>---</p>	3
O,Y	<p>J. CELL. BIOCHEM. SUPPL. vol. 14E, 1990, page 271; VISSER, R.G.F., ET AL.: 'Manipulation of starch in potatoes by new mutants and antisense RNA' see abstract R028</p> <p>---</p>	7
X	<p>MOL.GEN.GENET. vol. 225, no. 2, February 1991, pages 289 - 296; VISSER, R. G. F., ET AL.: 'Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs' see the whole document</p> <p>---</p>	1,4,5, 16,17
O,X	<p>THE PLANT CELL. vol. 3, no. 3, 1991, ROCKVILLE, MD, USA. pages 213 - 218; DILWORTH, M. F.: 'Molecular biology comes home' see page 216, right column, last paragraph - page 217, left column &amp; ORAL DISCLOSURE BY L.WILLMITZER, KEYSTONE SYMPOSIUM HELD JAN.10-17, 1991.</p> <p>---</p>	1,16,17
X	<p>ABSTRACTS VIITH INTERNATIONAL CONGRESS ON PLANT TISSUE CULTURE AND CELL CULTURE.ABSTRACT A5-36 1990, AMSTERDAM JUNE 24-29, 1990 page 177; VAN DER LEIJ, F.R.,ET AL.: 'Expression of the gene encoding granule bound starch synthase after introduction in an amylose-free and a wildtype potato (Solanum tuberosum)'</p> <p>---</p>	1,4,5, 16,17
X	<p>WO,A,9 012 876 (AKTIESELSKABET DANSKE SPRITFABRIKKER) 1 November 1990 see examples 24,25</p> <p>---</p>	1,4,5, 16,17
X	<p>WO,A,8 912 386 (CALGENE) 28 December 1989 see the whole document</p> <p>---</p>	1,4,16, 17

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	MOL. GEN. GENET. vol. 230, no. 1-2, November 1991, pages 39 - 44; KOSSMANN, J., ET AL.: 'Cloning and expression analysis of a potato cDNA that encodes branching enzyme: evidence for co-expression of starch biosynthetic genes'. see the whole document ---	1-3
P,X	EP,A,0 455 316 (INSTITUT GENBIOLOGISCHE FORSCHUNG BERLIN) 6 November 1991 see the whole document ---	1,4,5, 16,17

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9200302  
SA 56101

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 15/05/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9012084	18-10-90	US-A- 5034323	23-07-91
		AU-A- 5412390	05-11-90
		EP-A- 0465572	15-01-92
		WO-A- 9011682	18-10-90
WO-A-9012876	01-11-90	AU-A- 5531890	16-11-90
		EP-A- 0470145	12-02-92
WO-A-8912386	28-12-89	AU-A- 3852089	12-01-90
EP-A-0455316	06-11-91	DE-A- 4013144	24-10-91

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- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

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